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SHORT COMMUNICATION

J. W. Sanders · G. Venema · J. Kok · K. Leenhouts

Identification of a sodium chloride-regulated promoter in *Lactococcus lactis* by single-copy chromosomal fusion with a reporter gene

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Abstract An integration vector, pORI13, was developed to screen in *Lactococcus lactis* for expression signals induced by changes in the environment and to assay transcriptional activity of genes in single copy. The plasmid carries a promoterless *Escherichia coli lacZ* gene preceded by a start codon, a lactococcal ribosome binding site, and a multiple cloning site. Chromosomal *Sau*3AI fragments of *L. lactis* MG1363 DNA were cloned in pORI13 using a RepA$^+$ *E. coli* as host. The resulting bank of plasmids was used for Campbell-type integration into the chromosome of *L. lactis* MG1363. The relatively large size of the chromosomal fragments used increases the chance of retaining complete genes in the targeted region. Screening of integrants in the presence of 0.3 M NaCl resulted in the isolation of a clone (NS3) in which expression of *lacZ* was dependent on the concentration of chloride ions.

Key words Single copy transcriptional *lacZ* fusions · NaCl regulation · *Lactococcus lactis*

Introduction

A number of promoter-screening systems for *L. lactis* have been described, in which different reporter genes were used: *Escherichia coli lacZ* (Israelsen et al. 1995). *E. coli gusA* (Platteeuw et al. 1994), *L. lactis lacG* (Simons et al. 1990) and various chloramphenicol acetyltransferase (or *cat*) genes (Van der Vossen et al. 1985; Bojovic et al. 1991; Koivula et al. 1991). In most cases, these systems are plasmid based. Multiple copies of a regulated promoter on a plasmid may interfere with the regulation mechanism by titration of regulatory proteins. In addition, plasmid copy numbers may vary. Therefore, to assess the expression of a regulated gene accurately, transcriptional fusion to a single copy of a reporter gene in the chromosome is preferable. Recently, a Tn*917*-based promoter-screening system using *lacZ* as a reporter has been used to identify lactococcal promoters expressed under conditions of low temperature and low pH (Israelsen and Hansen 1993; Israelsen et al. 1995). Transposon insertion, however, results in many cases in gene disruption.

A new promoter-screening system was therefore developed, in which the Campbell-type integration vector pORI13 was used to search for environmentally regulated promoters. pORI13 is a member of the pORl family of integration vectors that can replicate only in helper strains providing *in trans* the RepA plasmid replication protein from the broad host range lactococcal plasmid pWV0l. The pORl vectors can integrate into the chromosome of host strains by recombination if RepA is absent and homology with the chromosome is provided. pORl vectors have been used for the generation of random insertion mutants and for the construction of targeted chromosomal deletions by replacement recombination (Law et al. 1995; Leenhouts et al. 1996). Here another variant, suitable for single-copy promoter screening and analysis is presented.

Results

In pMG60 (Table 1) *lacZ* of *E. coli* is fused to lactococcal translation signals from *orf-32* (Van der Vossen et al. 1987). This *lacZ* cassette was inserted into the integration vector pORl28 in a number of steps using the lactococcal RepA$^+$ helper strain LL108. The resulting plasmid was designated pORI13 (Fig. 1). It contains translational stop codons in all three reading frames, upstream of, or overlapping the start codon of *lacZ* to

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Table 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
L. lactis[a]		
MG1363	Plasmid-free derivative of NCDO712	Gasson (1983)
LL108	Cm^r, MG1363 derivative, carrying multiple *repA* copies in the chromosome	K. Leenhouts
LL302	MG1363 derivative, carrying a single *repA* copy in *pepX*	K. Leenhouts
NS3	Em^r, *lacZ* fused to the lactococcal NaCl-inducible transcription signal	This study
E. coli		
EC1000	Km^r, MC1000 derivative, carrying a single copy of pWV0l *repA* in *glgB*	Leenhouts et al. (1996)
Plasmids		
pMG60	Em^r, fusion of promoter 32 with *lacZ*	Van de Guchte et al. (1991)
pORI28	Em^r, *ori*$^+$, RepA$^-$ derivative of pWV0l	Leenhouts et al. (1996)
pORI13	Em^r, promoterless *lacZ*, *ori*$^+$, RepA$^-$ derivative of pWV01	This study
pVE6007	Cm^r, pWV01 derivative encoding a temperature-sensitive RepA protein	Maguin et al. (1992)
pNS3	Em^r, pORI13 carrying a 10-kb *Sau*3AI chromosomal DNA fragment, containing a NaCl-inducible transcription signal	This study
pNS3d	Em^r, NS3::*lacZ*, pORI13 carrying a 2.4-kb *Pst*I-*Sau*3AI chromosomal DNA fragment, deletion derivative of pNS3	This study
pNS3e	Em^r, NS3::*lacZ*, pORI13 carrying a 550-bp *Eco*RI-*Sau*3AI chromosomal DNA fragment, deletion derivative of pNS3	This study
pNS3f	Em^r, NS3::*lacZ*, pORI13 carrying a 450-bp *Xba*I-Sau3AI chromosomal DNA fragment, deletion derivative of pNS3	This study

[a] *L. lactis* was grown at 30° C in ½M17 broth, with a final concentration of 1.9% β-glycerolphosphate and 0.5% glucose. Erythromycin (Em) and chloramphenicol (Cm) were used at final concentrations of 5 μg/ml

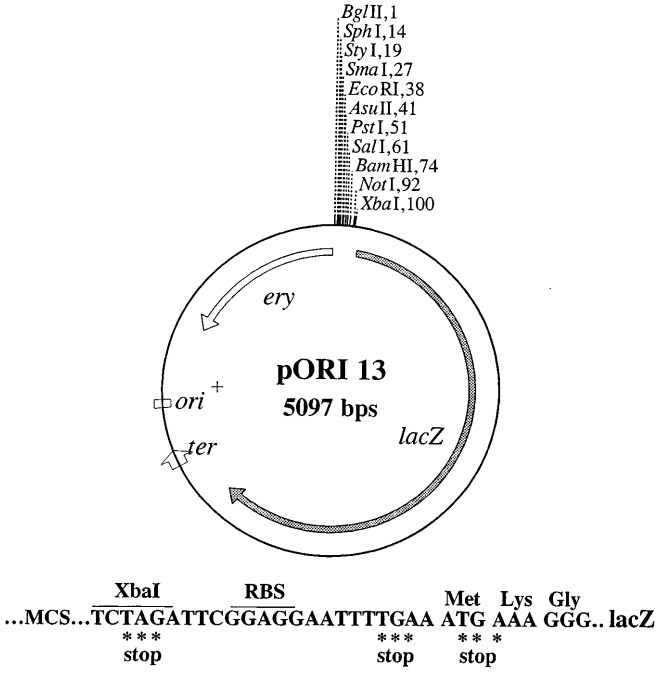

Fig. 1 Schematic representation of plasmid pORI13. *ori*$^+$, plus origin of replication of pWVO1; *ery*, erythromycin resistance gene; *ter*, terminator of *prtP*; *lacZ*, promoterless *E. coli* β-galactosidase gene fused to the ribosome binding site (RBS) and translational start codon of lactococcal *orf-32*; MCS, multiple cloning site. Stop codons are indicated by *asterisks*

prevent translational fusions. Plasmid pORI13 did not produce detectable β-galactosidase activity when allowed to replicate in *L. lactis* strains LL108 and LL302.

A genome bank in pORI13 was constructed by making a partial *Sau*3AI digest of total chromosomal DNA of *L. lactis* MG 1363 to obtain a majority of fragments ranging in size from 1 to 10 kb. These were ligated into the alkaline phosphatase-treated *Bam*HI restriction enzyme site of pORI13. The ligation mixture was used to transform *E. coli* EC1000 (RepA$^+$). Transformants were collected from agar plates by flooding each plate with 2 ml of TY broth, and their plasmid DNAs were isolated. The plasmid mixture was used to transform *L. lactis* MG1363 (pVE6007). Plasmid pVE6007 encodes a temperature-sensitive RepA protein. Integration of the pORI13 derivatives into the *L. lactis* chromosome was achieved by incubation of the transformants at 37°C as described by Law et al. (1995). In this way, Em^r colonies were obtained after plating onto GM17 agar containing 0.5 M sucrose and 0.008% X-gal, 2 μg/ml erythromycin and 0.3 M NaCl. Southern hybridisation analysis of 13 clones showed that pORI13 had integrated at 13 different positions in the chromosome of these clones (data not shown). One hundred and ninety-five colonies showed various levels of blue staining in the presence of NaCl and X-gal after prolonged incubation at 30° C, also indicative of *lacZ* insertion in different transcribed regions. These colonies were transferred to GM17-X-gal agar plates with or without 0.5 M NaCl. One clone selected, designated *L. lactis* NS3, produced blue colonies on plates with NaCl but was white on plates without added NaCl.

The integrated pORI13 derivative of NS3 (pNS3) was rescued as described (Law et al. 1995) and still showed the NaCl-dependent phenotype when present in the replicating form in *L. lactis* LL108. Restriction enzyme

analysis of pNS3 revealed that about 10 kb of chromosomal DNA was present upstream of *lacZ*. Digestion with *Pst*I, *Eco*RI and *Xba*I was used to map the NaCl-dependent promoter (Fig. 2). Deletions derivatives of NS3 were analysed on a plasmid in *L. lactis* LL108, with an intact copy of NS3 on the chromosome. The DNA region involved in NaCl-dependent gene expression is located between 540 and 440 bp upstream of the fusion point. The nucleotide sequence of this region was determined and is presented in Fig. 3. Two open reading frames (*orf*) were identified, separated by a 143 bp non-coding region. No known proteins similar to the deduced amino acid sequence of the 5′-*orf* were found. This *orfY* is directly followed by a 21-bp inverted repeat that may form a hairpin structure, functioning as a rho-independent terminator, with a calculated ΔG[25° C] of −35.0 kcal/mol (Tinoco et al. 1973). The 3′-truncated *orfX*, to which *lacZ* had been fused at codon 119, is preceded by a putative ribosome binding site and its deduced amino acid sequence is similar (69% of 119 residues are identical) to the N-terminal sequence of the putative amino acid antiporter GadC from *Shigella flexneri* (Waterman and Small 1996).

On the basis of the deletion studies and the nucleotide sequence, a NaCl-dependent start point of transcription was expected in the 100-bp region between the inverted repeat and the start of *orfX*. Primer-extension analysis was carried out using RNA isolated from LL108(pNS3d) grown in the absence or presence of 0.5 M NaCl (Fig. 4). Transcription started at an A residue 68 bp upstream of the AUG start codon of the

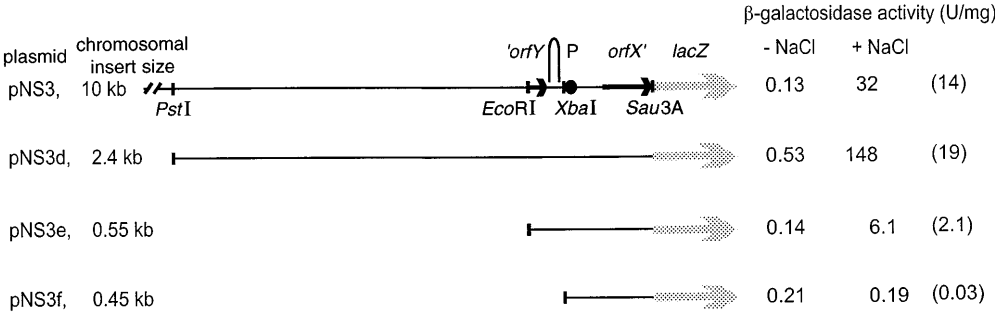

Fig. 2 Schematic representation of the chromosomal DNA fragment cloned in pNS3. NaCl-dependent transcriptional activity, as monitored by β-galactosidase activity in exponentially growing *L. lactis* LL108, is shown at the right. Deletion derivatives of pNS3 are shown schematically. The *lacZ* fusion point is identical in all these plasmids. β-Galactosidase activities in the absence or presence of NaCl are means of at least three independent experiments; standard derivations are given in parentheses. The background level of pORI13 is about 0.20 U/mg. *Stippled arrow*, *lacZ* reporter gene; *black arrow*, open reading frame; P, promoter. Only those restriction enzyme sites used for subcloning experiments are indicated

Fig. 3 Nucleotide sequence of the NS3 promoter region. The *vertical arrow* points to the transcription start point (shown in *lower case* letter. The −10 hexanucleotide is shown in *bold face* and is *underlined*. The location of the primer used for primer extension is also *underlined*. The *large horizontal arrows* show an inverted repeat that immediately follows the stop codon at positions 47–49 (*asterisks*). A ribosome binding site (rbs) is given in *lower case* and the deduced amino acid sequences of two truncated open reading frames are indicated. Relevant restriction enzyme sites are shown in *italics*. The *Sau*3AI site at the 3′-end of the sequence marks the fusion point with *lacZ* in pORI13. The GenBank accession number is AF005098

```
                                  EcoRI    'orfY
  1                               GAA TTCTATCAGA ATAAAATGGA AAAGTATTTG AAAAGGTCAA
                                   E   F  Y  Q   N  K  M   E  K  Y  L   K  R  S

 44 TTTAGTCTCT TTTGAGCTGT TGCTTTAAAG CAACAGCTCA AAAGAGATTT TCTTTATTCT
    I ***----------------------><----------------------

   XbaI      -10            ↓
104 AGAGCATATA CTAGAGGGTG aAGATAGGTT GTCTGAAGCA TTATAACTTG TCTTTTAAAA

                                        start orfX
164 AATTCAATCA TAAATATaag gaggtATGAT GAATCAAAAA AAATTATCAT TATTCGGTTT
  1                        rbs       M   N  Q  K   K  L  S   L  F  G  F
     Primer NS3-11
224 TTTCGCATTA ACCGCTTCAA TGGTTTTGAC TGTCTATGAG TATCCGACTT TTGCCACGTC
 12   F  A  L   T  A  S   M  V  L  T   V  Y  E   Y  P  T   F  A  T  S

284 AAAATTACAT TTGGTGTTCT TTTTACTTCT CGGAGGACTA CTATGGTTTT TGCCTGTAGC
 32   K  L  H   L  V  F  F   L  L  L   G  G  L   L  W  F   L  P  V  A

344 GCTCTGCGCA GCAGAAATGG CGACGGTTGA AGGCTGGAAA AATGGTGGAA TCTTTAGTTG
 52   L  C  A   A  E  M   A  T  V  E   G  W  K   N  G  G   I  F  S  W

404 GGTCAGTCAA ACTTTAGGTG AGCGCTTTGG TTTTGCAGCC ATATTTTTTC AGTGGTTCCA
 72   V  S  Q   T  L  G   E  R  F  G   F  A  A   I  F  F   Q  W  F  Q
                                  Sau3A
464 AATTACAGTA GGTTTTGTCA CTATGATCTA TTTCATTTTA GGGGCCCTCT CTTATGTGTT
 92   I  T  V   G  F  V   T  M  I   Y  F  I  L   G  A  L   S  Y  V  L
                               Sau3A
524 AAATTTTCAG GCGCTCAATA CAGATC   pORI13
112   N  F  Q   A  L  N   T  D      lacZ
```

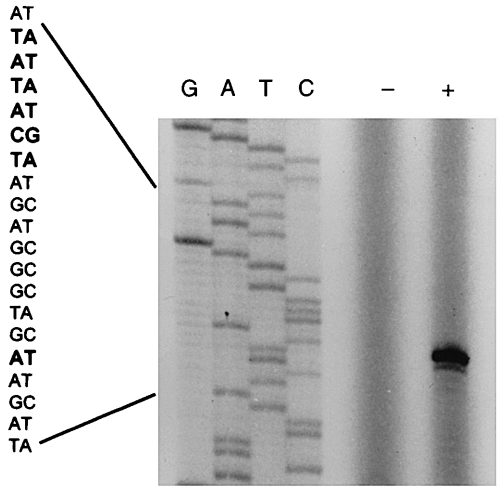

Fig. 4 Determination of the transcriptional start point of the NaCl-inducible transcript in NS3. Primer extension products were synthesised by using RNA from exponentially growing *L. lactis* LL108(pNS3d) (OD_{600} of 0.5), cultured with (+) or without (–) 0.5 M NaCl. RNA was isolated as previously described (Van Asseldonk et al. 1993). Some 25 ng of synthetic oligonuclotide NS3-11 (5′-CAGTCAAAACCATTGAAGCGGTTAATGCGAAAAAA-CCG) was added to 5 µg of RNA in a 5.5-µl reaction mixture containing 0.25 mM dCTP, 0.25 mM dGTP, 0.25 mM dTTP and 2 µM α-[^{35}S]dATP, and cDNA was synthesised using AMV reverse transcriptase (Boehringer Mannheim GmbH, Mannheim, Germany). After 10 min of incubation at 42° C an excess of cold dATP was added and incubation was continued for another 10 min at 42° C. The primer extension product was analysed on a sequencing gel next to the products of a DNA sequence reaction carried out using the same primer and pNS3 template DNA. The sequences of the transcribed and the sequenced strands are indicated. The −10 region of the promoter and the transcription start point are indicated in bold face

L. lactis orfX (position 141 in Fig. 3). No primer-extension product was obtained with RNA from cells grown in the absence of NaCl. The same NaCl-dependent start point was observed using RNA isolated from *L. lactis* MG1363 (not shown). Nine base pairs upstream of the transcription start point a −10 hexanucleotide was identified that differs in only 1 nucleotide from the consensus sequence (Van de Guchte et al. 1992). Further upstream, no −35-like hexanucleotide could be discerned. Instead, a 21-bp inverted repeat is located 19 bp upstream of the −10 hexanucleotide, suggesting that this structure may have a role in gene expression.

A number of compounds were tested for their ability to induce *lacZ* expression in *L. lactis* NS3 (Table 2). Since high levels of sucrose or glycerol in the growth medium resulted in low levels of enzyme activity, high osmolarity did not induce β-galactosidase expression. High levels of *lacZ* expression were obtained in the presence of chloride ions, while little or no *lacZ* expression was detectable in the presence of other ions. No induction of β-galactosidase activity was observed on increasing or decreasing the growth temperature. Interestingly, β-galactosidase activity increased logarithmically with NaCl concentrations in medium containing increasing concentrations of NaCl from 0 to 500 mM (data not shown).

Discussion

A reporter integration strategy was developed to identify gene expression signals in the chromosome of *L. lactis*. This strategy has the advantage that the expression signals can be monitored in a single-copy situation. The relatively large size of the chromosomal fragments used for integration increases the probability that, upon integration of pORI13, in addition to the disrupted copy, a wild-type version is retained in the transformed strain. This may imply that also two copies of a promoter and a target site for regulation are present in such a strain. An additional advantage of the strategy is that regulatory elements upstream of the fragment required for Campbell integration can be traced. After integration at the 3′-end of an operon, *lacZ* will come under the control of a promoter upstream of the fragment used for recombination. The host/vector system described here is suitable

Table 2 *lacZ* Expression in *L. lactis* LL108(pNS3d) in the presence of various compounds

Compound[a]	Concentration (mM)	β-Galactosidase activity (U/mg)[b]
None	–	1.1 (0.58)
NaCl	300	151 (28)
Sucrose	500	5.1 (3.8)
Glycerol	500	1.2 (0.45)
$MgSO_4$	300	3.3 (2.3)
KNO_3	300	7.7 (2.6)
Na_2SO_4	300	2.1 (0.69)
KCl	300	110 (36)
NH_4Cl	300	72 (33)
$MgCl_2$	150	133 (35)
NaI	300	4.3 (2.5)
KI	300	1.3 (0.89)

[a] Cultures of LL108(pNS3d) were grown to an absorbance (at 600 nm) of 0.5 in GM17 containing the indicated final concentration of a compound

[b] Cell extracts were prepared as described by Van de Guchte et al. (1992), β-Galactosidase activity was determined as described by Miller (1972). Protein concentrations were determined by the method of Bradford (1976). Values are means of at least three independent experiments; standard deviations are given in parentheses

both for single-copy screening of regulated promoters and for the construction of targeted transcriptional fusions without gene disruption (Sanders et al. 1995), and can most probably be used in many different bacterial species.

The NaCl-inducible expression from the NS3 promoter is conferred by chloride ions and is independent of the osmolarity, ionic strength or sodium ion concentration in the medium. To our knowledge, this is the first observation of gene regulation controlled by Cl^-. Induction of transcription by other ions, like Na^+, has been described (Dover et al. 1996). The mechanism by which Cl^- induces transcription from this promoter is not known. The lower level of NaCl-dependent *lacZ* expression from pNS3e compared to pNS3d suggests the presence of cis-acting elements upstream of the promoter. On the basis of similarity, the gene transcribed from the NaCl-dependent promoter is probably *gadC*. In *S. flexneri gadC* is involved in glutamate-dependent acid resistance (Waterman and Small 1996). The regulation of Cl− dependent transcription and its putative relationship to acid resistance are currently being investigated.

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References

Bojovic B, Djordjevic G, Topisirovic L (1991) Improved vector for promoter screening in lactococci. Appl Environ Microbiol 57:385–388

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein, utilizing the principle of protein-dye binding. Anal Biochem 12:248–254

Dover N, Higgins CF, Carmel O, Rimon A, Pinner E, Padan E (1996) Na^+-induced transcription of *nhaA*, which encodes an Na^+/H^+ antiporter in *Escherichia coli*, is positively regulated by *nhaR* and affected by *hns*. J Bacteriol 178:6508–6517

Gasson MJ (1983) Plasmid complements of *Streptococcus lactis* NCDO712 and other lactic streptococci after protoplast-induced curing. J Bacteriol 154:1–9

Israelsen H, Hansen EB (1993) Insertion of transposon Tn*917* derivatives into the *Lactococcus lactis* subsp. *lactis* chromosome. Appl Environ Microbiol 59:21–26

Israelsen H, Madsen SM, Vrang A, Hansen EB, Johansen E (1995) Cloning and partial characterization of regulated promoters from *Lactococcus lactis* Tn*917-lacZ* integrants with the new promoter probe vector pAK80. Appl Environ Microbiol 61:2540–2547

Law J, Buist G, Haandrikman A, Kok J, Venema G, Leenhouts K (1995) A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. J Bacteriol 177:7011–7018

Leenhouts KJ, Buist G, Bolhuis A, ten Berge A, Kiel J, Mierau I, Dabrowska M, Venema G, Kok J (1996) A general system for generating unlabeled gene replacements in bacterial chromosomes. Mol Gen Genet 253:217–224

Koivula T, Sibakov M, Palva I (1991) Isolation and characterization of *Lactococcus lactis* subsp. *lactis* promoters. Appl Environ Microbiol 57:333–340

Maguin E, Duwat P, Hege T, Ehrlich D, Gruss A (1992) New thermosensitive plasmid for gram-positive bacteria. J Bacteriol 174:5633–5638

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Platteeuw C, Simons G, de Vos WM (1994) Use of the *Escherichia coli* β-glucuronidase (*gusA*) gene as a reporter gene for analyzing promoters in lactic acid bacteria. Appl Environ Microbiol 60:587–593

Sanders JW, Leenhouts KJ, Haandrikman AJ, Venema G, Kok J (1995) Stress response in *Lactococcus lactis*: cloning, expression analysis, and mutation of the lactococcal superoxide dismutase gene. J Bacteriol 177:5254–5260

Simons G, Buys H, Hogers R, Koenhen E, de Vos WM (1990) Construction of a promoter-probe vector for lactic acid bacteria using the *lacG* gene of *Lactococcus lactis*. J Indust Microbiol 31:31–39

Tinoco I Jr, Borer PN, Dengler B, Levine MD, Uhlenbeck OC, Crothers DM, Gralla J (1973) Improved estimation of secondary structure in ribonucleic acids. Nature 246:40–41

Van Asseldonk M, Simons A, Visser H, de Vos WM, Simons G (1993) Cloning, nucleotide sequence, and regulatory analysis of the *Lactococcus lactis dnaJ* gene. J Bacteriol 175:1637–1644

Van de Guchte M, Van der Lende T, Kok J, Venema G (1991) Distance-dependent translational coupling and interference in *Lactococcus lactis*. Mol Gen Genet 227:65–71

Van de Guchte M, Kok J, Venema G (1992) Gene expression in *Lactococcus lactis*. FEMS Microbiol Rev 88:73–92

Van der Vossen JMBM, Kok J, Venema G (1985) Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for *Bacillus subtilis* and *Streptococus lactis*. Appl Environ Microbiol 50:540–542

Van der Vossen JMBM, van der Lelie D, Venema G (1987) Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. Appl Environ Microbiol 53:2452–2457

Waterman SR, Small PLC (1996) Identification of σ^s-dependent genes associated with the stationary phase acid-resistance phenotype of *Shigella flexneri*. Mol Microbiol 21:925–940